Salinispora arenicola gen. nov., sp. nov. and Salinispora tropica sp. nov., obligate marine actinomycetes belonging to the family Micromonosporaceae

Luis A. Maldonado,† William Fenical, Paul R. Jensen, Christopher A. Kauffman, Tracy J. Mincer, Alan C. Ward, Alan T. Bull and Michael Goodfellow

1School of Biology, University of Newcastle, Newcastle upon Tyne NE1 7RU, UK
2Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, CA 92093-0204, USA

A taxonomic study was carried out to clarify the taxonomy of representatives of a group of marine actinomycetes previously designated MAR 1 and considered to belong to the family Micromonosporaceae. The organisms had phenotypic properties consistent with their assignment to this taxon. The strains formed a distinct taxon in the 16S rRNA Micromonosporaceae gene tree and shared a range of phenotypic properties that distinguished them from members of all of the genera with validly published names classified in this family. The name proposed for this novel taxon is Salinispora gen. nov. The genus contains two species recognized using a range of genotypic and phenotypic criteria, including comparative 16S–23S rRNA gene spacer region and DNA–DNA relatedness data. The names proposed for these taxa are Salinispora arenicola sp. nov., the type species, and Salinispora tropica sp. nov.; the type strains of these novel species have been deposited in service culture collections as strain CNH-643 (= ATCC BAA-917 = DSM 44819) and strain CNB-440 (= ATCC BAA-916 = DSM 44818), respectively.

INTRODUCTION

The family Micromonosporaceae Krasil’nikov 1938 emend. Stackebrandt et al. 1997 is a member of the suborder Micromonosporineae, one of the taxa which make up the order Actinomycetales Buchanan 1917 emend. Stackebrandt et al. 1997. The emended family is phylogenetically distinct but encompasses a chemotaxonomically and morphologically diverse group of filamentous organisms belonging to the genera Actinoplanes, Catellatospora, Catenuloplanes, Couchioplanes, Dactylosporangium, Micromonospora and Pilimelia (Koch et al., 1996). The genera Asanoa, Lee and Hah 2002, Longispora Matsumoto et al. 2003, Spirilliplanes Tamura et al. 1997, Verrucosispora Rheims et al. 1998 and Virgisporangium Tamura et al. 2001 were subsequently added to the family. Members of all of the genera classified in the family Micromonosporaceae can be distinguished from one another using a combination of chemical and morphological properties (Lee & Hah, 2002; Matsumoto et al., 2003).

Actinomycetes are generally considered to be indigenous to terrestrial habitats, although it is becoming increasingly apparent that they are common in marine ecosystems (Bull et al., 2000; Maldonado et al., 2005), as exemplified by the isolation of corynebacteria, dietzias, gordoniae, mycobacteria and rhodococci from deep-sea sediments (Colquhoun et al., 1998; Takami et al., 1999), and micromonosporaceae and streptomycetes from bathyal and coastal sediments (Goodfellow & Haynes, 1984; Jensen et al., 1991; Takizawa et al., 1993). There is convincing evidence that actinomycetes are adapted to marine habitats (Moran et al., 1995; Mincer et al., 2002), though to date only one marine genus, Salinibacterium Han et al. 2003, and three marine species, Dietzia maris (Nesterenko et al. 1982) Rainey et al. 1995, Rhodococcus marinonascens Helmke and Weyland 1984 and Williamsia maris Stach et al. 2004, have been described.
However, it is evident from analyses of community DNA that current culture-based techniques grossly underestimate the diversity of actinomycetes in estuarine and marine sediments (Stach et al., 2003a, b; Piza et al., 2004). There is evidence that marine actinomycetes produce novel metabolites of commercial interest (Felgen et al., 2003; Fiedler et al., 2005), as shown by the ability of a marine Verrucosispora strain to produce inhibitors of the paraaminobenzoic acid pathway (Riedlinger et al., 2004).

The first conclusive evidence for the widespread and persistent occurrence of indigenous actinomycete populations in marine sediments was reported by Mincer et al. (2002), who isolated large numbers of strains, designated MAR 1, from geographically diverse tropical and/or subtropical locations. The strains were distinguished by morphological characteristics, small-subunit rRNA gene signature nucleotides and by an obligate requirement for sea water for growth. Phylogenetic analyses of nearly complete 16S rRNA gene sequences of seven strains showed that they formed a monophyletic clade within the family Micro- monosporaceae that suggested novelty at the genus level. The MAR 1 isolates were provisionally assigned to a taxon informally designated ‘Salinispora’ (Mincer et al., 2002; Felgen et al., 2003).

The aim of the present study was to establish the taxonomic status of representative MAR 1 isolates by using a polyphasic approach. The resultant data show that the tested strains form a novel taxon, Salinispora gen. nov., which encompasses two novel species, Salinispora arenicola sp. nov. and Salinispora tropica sp. nov.

**METHODS**

**Isolation, maintenance and cultivation.** The representative MAR 1 strains (isolates CNB-440 T, CNB-536, CNH-643 T, CNH-646, CNH-898 and CNH-964) were recovered from marine sediments, as described by Mincer et al. (2002). The organisms were maintained on glucose/yeast/malt extract agar (ISP 2; Shirling & Gottlieb, 1966) at room temperature and as glycerol suspensions (20 %, v/v) at 20 °C. Biomass for the chemical and molecular systematic studies was grown in shake flasks of ISP 2 broth (Shirling & Gottlieb, 1966) for 10 days at 28 °C, and then harvested by centrifugation. Cells for the chemosystematic studies were washed in distilled water and freeze-dried; those for the molecular systematic work were washed in NaCl/EDTA buffer (0·1 M EDTA, pH 8·0, 0·1 M NaCl) and stored at −20 °C until required. All media were prepared with artificial sea water (Aquarium Systems: http://www.aquariumsystems.com) to give a final concentration of 3·5 % (w/v) NaCl.

**Cultural and morphological studies.** The cultural properties of the strains were observed on ISP 2 agar (Shirling & Gottlieb, 1966) and modified Bennett’s agar (Jones, 1949) supplemented with mannitol (0·5 %, w/v), soybean flour (0·5 %, w/v) and NaCl (3·5 %, w/v), following incubation at 28 °C for 4 weeks. Spore arrangement and spore surface ornamentation were observed by examining gold-coated, dehydrated material, prepared from 14-day-old cultures grown on modified Bennett’s agar at 28 °C, using a Hitachi 5·570 scanning electron microscope. Gram (Hucker’s modification; Society of American Bacteriologists, 1957) and Ziehl–Neelsen (Gordon, 1967) preparations were examined by light microscopy following growth on ISP 2 and modified Bennett’s agar plates.

**Biochemical and physiological properties.** The strains were examined for their ability to degrade a range of organic compounds by using established methods (Williams et al., 1983) following incubation at 28 °C for 21 days, and for their capacity to grow on a range of sole carbon compounds after 14 days at 28 °C, following the procedure described by Pridham & Gottlieb (1948). Tolerance to pH, temperature and NaCl concentrations was recorded on ISP 2 agar (Shirling & Gottlieb, 1966) following growth at 28 °C for 4 weeks. Resistance to antibiotics was determined following incubation at 28 °C for 7, 14 and 21 days using ISP 2 agar (Shirling & Gottlieb, 1966) as the basal medium.

**Chemotaxonomy.** Standard HPLC and TLC procedures were used to determine the isomers of diaminopimelic acid (Stanek & Roberts, 1974), fatty acid profiles (Komagata & Suzuki, 1987), predominant menaquinones (Collins, 1994), whole-organism sugars (Schaal, 1985) and muramic acid type (Uchida et al., 1999).

**Phylogeny.** The almost-complete 16S rRNA gene nucleotide sequences of the six strains, as generated by Mincer et al. (2002), were aligned manually with corresponding sequences of representatives of all of the genera currently classified in the family Micro- monosporaceae retrieved from the DDBJ/EMBL/GenBank databases, by using the PHYDIT program (available at http://plaza.snu.ac.kr/~jchun/phydit/). Evolutionary trees were inferred using the least-squares, maximum-likelihood, maximum-parsimony and neighbour-joining tree-making algorithms from the PHYLIP suite of programs (Felsenstein, 1993) and evolutionary distance matrices generated for the least-squares and neighbour-joining methods, as described by Jukes & Cantor (1969). The topologies of the resultant trees were evaluated by bootstrap analyses (Felsenstein, 1985) of the neighbour-joining dataset based on 1000 resamplings, using the SEQBOOT and CONSENSE options from the PHYLIP package (Felsenstein, 1993).

**DNA base composition and DNA–DNA relatedness studies.** A standard procedure was used to extract genomic DNA from each of the test strains (Pitcher et al., 1989). DNA from strains CNB-440 T and CNH-643 T was digested, dephosphorylated and analysed using a reversed-phase HPLC procedure (Tamaoka, 1994), as described by Kim et al. (1998), and molar G+C content was calculated after Mesba et al. (1989). DNA–DNA relatedness studies were carried out between strain CNB-440 T and strains CNB-536 and CNH-898, between strain CNH-643 T and strains CNH-646 and CNH-964, and between strains CNB-440 T and CNH-643 T, using the identification service at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany), as described by Kim et al. (1999).

**Phylogenetic analysis based on 16S rRNA gene spacer sequence comparisons.** PCR of intergenic spacer regions of the test strains was carried out as described by Zhang et al. (2001). The resultant PCR products were purified by using the Wizard PCR purification system (Promega), and then sequenced using DyeDeoxy Terminator Cycle sequencing kits (Applied Biosystems) and the complementary sequence of the universal primer 1525r described by Lane (1991). The resultant sequences were aligned manually against available corresponding sequences of members of the genera Micro- monospora and Verrucosispora retrieved from the DDBJ/EMBL/ GenBank databases. Evolutionary trees were inferred using the neighbour-joining tree-making algorithm from the PHYLIP suite of programs (Felsenstein, 1993) and an evolutionary distance matrix generated following Jukes & Cantor (1969).
RESULTS AND DISCUSSION

It was apparent from the present study that the six representative MAR 1 strains have properties consistent with their assignment to the family *Micromonosporaceae* (Goodfellow et al., 1990; Koch et al., 1996; Tamura et al., 1997). To this end, all of the strains were aerobic, Gram-positive, non-acid-fast actinomycetes that formed a branched substrate mycelium and non-motile spores borne singly or in clusters, and contained *meso*-diaminopimelic acid and *N*-glycolylated muramic acid in the wall peptidoglycan, arabinose, galactose and xylose as major sugars, diphasatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol and phosphatidylinositol as diagnostic phospholipids, tetrahydrogenated menaquinones with nine isoprene units as the major isoprenologue, and complex mixtures of saturated, iso- and anteiso-fatty acids, but lacked mycolic acids.

The 16S rRNA gene sequence data confirmed and extended earlier results reported by Mincer et al. (2002) in showing that the MAR 1 strains formed a distinct monophyletic clade that corresponded to phyletic lines formed by representatives of the 12 genera with validly published names currently classified in the family *Micromonosporaceae* (Fig. 1). The
status of the MAR 1 clade was supported by data from all of the tree-making algorithms and by a bootstrap value of 100 % in the neighbour-joining analysis. It was also evident that the representative MAR 1 strains had a phenotypic profile that separated them from members of all of the genera classified in the family Micromonosporaceae (Table 1). Indeed, the abundance, widespread distribution, phylogenetic divergence and physiological adaptations of the MAR 1 isolates suggest that they represent an ecologically significant component of the bacterial community in tropical marine sediments (Mincer et al., 2002). The present data provide yet further evidence for recognizing that MAR 1 strains represent a new centre of taxonomic variation in the family Micromonosporaceae that merits generic status.

The 16S rRNA genes of the six representative MAR 1 isolates were relatively highly conserved as they shared similarities within the range 98–99.9 %, values which correspond to 2 and 17 nucleotide differences, respectively, at 1480 locations. Nevertheless, it is evident from Fig. 1 that the six strains can be assigned to two, albeit closely related, groups supported by high bootstrap values. In cases such as this, nucleotide sequences of alignable stretches of DNA that are less conserved than those of corresponding 16S rRNA gene nucleotide sequences of alignable stretches of DNA that are supported by high bootstrap values. In cases such as this, nucleotide sequences of alignable stretches of DNA that are less conserved than those of corresponding 16S rRNA gene sequences, such as sequence heterogeneity in 16S rRNA gene spacer regions, can be used to highlight differences between strains (Gürtler & Stanisich, 1996; García-Martínez et al., 2001; Zhang et al., 2001). It is clear from Fig. 2 that the current 16S rRNA gene spacer sequence data provide a much better resolution of the two taxa delineated in the 16S rRNA gene study; the taxonomic integrity of the two sharply delineated groups was supported by high bootstrap values. However, the two groups merge to form a taxon that is distinct from corresponding groups containing representatives of the genera Micromonospora and Verrucosispora.

DNA–DNA relatedness studies provide a reliable way of distinguishing between representatives of actinomycete species that share high 16S rRNA gene similarities (Nam et al., 2003; Jones et al., 2004; Groth et al., 2004). It is also well known that the minimum level of DNA–DNA relatedness required to circumscribe genomic species is taken to be 70 % (Wayne et al., 1987). It was apparent from the DNA–DNA relatedness data that the members of the two phylogenetic subclades belong to distinct genomic species. Strains CNB-536 and CNH-898 shared similarity values of 99.8 and 88.8 % with reference DNA from strain CNB-440\(^T\), whereas strains CNH-646 and CNH-964 had corresponding values of 84.4 and 86.2 % with strain CNH-643\(^T\); strains CNB-440\(^T\) and CNH-643\(^T\) had a DNA–DNA relatedness

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>Require sea water for growth</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Spore motility</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Spore vesicles</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Wall chemotype*</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>VI</td>
<td>VI</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
</tr>
<tr>
<td>Fatty acid type†</td>
<td>3a</td>
<td>2d</td>
<td>2d</td>
<td>2d</td>
<td>2d</td>
<td>2d</td>
<td>2d</td>
<td>2d</td>
<td>2d</td>
<td>2d</td>
<td>2d</td>
<td>2d</td>
<td>2d</td>
</tr>
<tr>
<td>Major menaquinones (MK-*)</td>
<td>9(H(_4))</td>
<td>9(H(_4))</td>
<td>10(H(_4))</td>
<td>10(H(_4))</td>
<td>9(H(_4), H(_6))</td>
<td>11(H(_4))</td>
<td>9(H(_4), H(_6))</td>
<td>10(H(_4), H(_6))</td>
<td>9(H(_4), H(_6))</td>
<td>10(H(_4), H(_6))</td>
<td>9(H(_4), H(_6))</td>
<td>10(H(_4), H(_6))</td>
<td>9(H(_4), H(_6))</td>
</tr>
<tr>
<td>Phospholipid type‡</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>III</td>
<td>II</td>
<td>III</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
<td>II</td>
</tr>
<tr>
<td>G+C content (mol%)</td>
<td>70–73</td>
<td>72–73</td>
<td>71–72</td>
<td>70–72</td>
<td>70–73</td>
<td>72–73</td>
<td>72–73</td>
<td>70</td>
<td>71–73</td>
<td>ND</td>
<td>69</td>
<td>70</td>
<td>71</td>
</tr>
</tbody>
</table>

*According to the classification of Lechevalier & Lechevalier (1970).
†According to the classification of Kroppenstedt (1985).
‡According to the classification of Lechevalier et al. (1977).
value of 44·9%. Members of these genomic species could be distinguished by using a set of phenotypic properties. Strains CNH-643T, CNH-646 and CNH-964, unlike the remaining strains, utilized L-proline, L-threonine, (+)-D-salicin and L-tyrosine as sole carbon sources and grew in the presence of rifampicin (25 μg ml⁻¹). Similarly, only strains CNB-440T, CNB-536 and CNH-898 utilized (+)-D-galactose and inulin as sole carbon sources.

The genotypic and phenotypic data acquired in the present study, taken together with complementary data from Mincer et al. (2002), show that the six representative MAR 1 isolates form a new centre of taxonomic variation within the family Micromonosporaceae Krasil’nikov 1938 emend. Stackebrandt et al. 1997. The name proposed for this taxon is Salinispora gen. nov. Similarly, two species can be recognized in this genus, Salinispora arenicola sp. nov., the type species, and Salinispora tropica sp. nov.

Description of Salinispora gen. nov.

Salinispora (Sa.li.ni.spo’ra. N.L. adj. salinus saline; Gr. fem. n. spora a seed and, in bacteriology, a spore; N.L. fem. n. Salinispora a spore-forming bacterium originating from a saline habitat, indicating the marine habitat of the organism).

The description is based upon information taken from this study and from Mincer et al. (2002). Aerobic, Gram-positive, non-acid-fast actinomycetes that form extensively branched substrate hyphae (0·25–0·5 μm in diameter) that carry smooth-surfaced spores (0·8–3·8 μm in diameter) singly or in clusters (Fig. 3). All strains require sea water or a sodium-supplemented medium for growth. Colonies first appear within 3–6 weeks depending on the growth medium. They are recognized by their lack of aerial hyphae and their pigmentation; the latter ranges from bright to pale orange to black on M1 medium. Spreading substrate mycelia are formed on low-nutrient media M4 and M5. Dark brown to black, bright orange or pink diffusible pigments are frequently produced. Colonies can become darkened during sporulation, with spores borne either sessily or on short sporophores. Vegetative hyphae are finely branched and do not fragment. Good growth occurs at 10–30°C and pH 7–12. Arbutin (0·1%, w/v), casein (1%, w/v), elastin
Description of *Salinispora arenicola* sp. nov.

*Salinispora arenicola* [ar.en.i.co’en] l. n. arena sand; L. suff. -cola (from L. n. incola) inhabitant, dweller; N.L. n. arenicola sand-dweller, indicating isolation from marine sediments.

The description is based on information taken from this study and from Mincer *et al.* (2002). Morphological, chemotaxonomic, genomic and general characteristics are as given in the genus description. The temperature range for growth is 10–30 °C, with an optimum at 15–28 °C. (+)-D-Galactose and inulin are used as sole carbon sources for energy and growth. Does not grow in the presence of rifampicin (25 µg ml⁻¹). Isolated from coarse sand off the Bahamas. The type strain is CNB-440T (= ATCC BAA-916T = DSM 44818T).

**ACKNOWLEDGEMENTS**

A.T.B., M.G., L.A.M. and A.C.W. are indebted to the UK Natural Environmental Research Council for support (grants NER/T/S/2000/00614 and NER/T/S/2000/00616). W.F. is grateful for funding from the National Institutes of Health, National Cancer Institute (grant CA44848) and the University of California Industry–University Cooperative Research Program (IUCRP; grant BioStar 10102). P.R.J. and W.F. acknowledge that they were scientific advisors to the corporate sponsor of the IUCRP award. The terms of this arrangement have been reviewed and approved by the University of California, San Diego, in accordance with its conflict of interest policies.

**REFERENCES**


L. A. Maldonado and others


